Biochimica et Biophysica Acta 1808 (2011) 2428–2434

Contents lists available at ScienceDirect

Biochimica et Biophysica Acta

journal homepage: www.elsevier.com/locate/bbamem

Desmosterol replaces cholesterol for ligand binding function of the serotonin$_{1A}$ receptor in solubilized hippocampal membranes: Support for nonannular binding sites for cholesterol?

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ARTICLE INFO

Article history:
Received 14 April 2011
Received in revised form 25 June 2011
Accepted 27 June 2011
Available online 5 July 2011

Keywords:
Serotonin$_{1A}$ receptor
Cholesterol
Desmosterol
Ligand binding function
Fluorescence anisotropy
Nonannular binding site

ABSTRACT

The serotonin$_{1A}$ receptor is an important member of the G-protein coupled receptor family, and is involved in the generation and modulation of a variety of cognitive and behavioral functions. Solubilization of the hippocampal serotonin$_{1A}$ receptor by CHAPS is accompanied by loss of cholesterol that results in a reduction in specific agonist binding activity. Replenishment of cholesterol to solubilized membranes restores membrane cholesterol content and significantly recovers specific agonist binding. In order to test the stringency of cholesterol requirement, we solubilized native hippocampal membranes followed by replenishment with desmosterol. Desmosterol is the immediate biosynthetic precursor of cholesterol in the Bloch pathway differing only in a double bond at the 24th position. Our results show that replenishment with desmosterol restores ligand binding of serotonin$_{1A}$ receptors. This is consistent with earlier results showing that desmosterol can replace cholesterol in a large number of cases. However, these results appear to be contradictory to our earlier findings, performed by sterol manipulation utilizing methyl-β-cyclodextrin, in which we observed that replacing cholesterol with desmosterol is unable to restore specific ligand binding of the hippocampal serotonin$_{1A}$ receptor. We discuss the possible molecular mechanism, in terms of nonannular lipid binding sites around the receptor, giving rise to these differences.

1. Introduction

Cholesterol is an essential and representative lipid in higher eukaryotic cellular membranes and is crucial in membrane organization, dynamics, function, and sorting [1,2]. It is often found distributed nonrandomly in domains in biological and model membranes [3–6]. These domains (sometimes termed as 'lipid rafts') contribute to variable patchiness of the membrane, and are believed to be important since membrane sorting and trafficking [7], signal transduction processes [8], and the entry of pathogens [9] have been attributed to these type of domains.

Cholesterol is the end product of a long, multi-step and exceedingly fine-tuned sterol biosynthetic pathway that parallels sterol evolution [10,11]. Konrad Bloch speculated that the sterol biosynthetic pathway parallels sterol evolution (the "Bloch hypothesis"). According to this hypothesis, cholesterol has been selected over a very long time scale of natural evolution for its ability to optimize certain physical properties of eukaryotic cell membranes with regard to biological functions. Desmosterol is the immediate precursor of cholesterol in the Bloch pathway of cholesterol biosynthesis and differs with cholesterol only in a double bond at the 24th position in its flexible alkyl side chain (see Fig. 1). Desmosterol is converted to cholesterol in the final step of the Bloch pathway by the enzyme 3β-hydroxy-steroid-Δ^{24}-reductase (DHCR24). Interestingly, it has been previously reported that *Dhcr24* gene knockout (*Dhcr24*$^{-/-}$) mice, which contain desmosterol instead of cholesterol, are viable and exhibit a mild phenotype [12].

The G-protein coupled receptor (GPCR) superfamily is the largest and most diverse protein family in mammals, involved in signal transduction across membranes [13,14]. GPCRs are prototypical members of the family of seven transmembrane domain proteins and include >800 members which together constitute ~5% of the human genome [15]. GPCRs regulate physiological responses to a diverse array of stimuli, and mediate multiple physiological processes. For this reason, GPCRs have emerged as major targets for the development of novel drug candidates in all clinical areas [16]. It is estimated that up to 50% of clinically prescribed drugs act as either agonists or antagonists of GPCRs [17]. The serotonin$_{1A}$ receptor is an important member of the GPCR family and is implicated in the generation and modulation of various cognitive, behavioral and developmental functions [18–20]. The serotonin$_{1A}$ receptor agonists and antagonists have been shown to possess potential therapeutic effects in anxiety or stress-related disorders [18]. As a result, the serotonin$_{1A}$ receptor serves as an important target in the development of therapeutic agents for neuropsychiatric disorders such as anxiety

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doi:10.1016/j.bbamem.2011.06.022

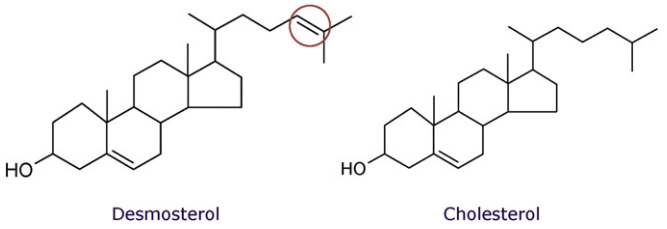

Fig. 1. Chemical structures of desmosterol and cholesterol. Desmosterol is the immediate precursor of cholesterol in the Bloch pathway, one of the two routes of cholesterol biosynthesis. It differs with cholesterol only in a double bond at the 24th position in the flexible alkyl side chain (highlighted in its chemical structure). See text for more details.

and depression. In view of the increasing pharmacological relevance of the serotonin$_{1A}$ receptor, a transmembrane protein, its interaction with the surrounding membrane lipids assumes significance in modulating the function of the receptor in healthy and diseased states [20,21]. In this context, previous work from our laboratory comprehensively demonstrated the requirement of membrane cholesterol in the function of the serotonin$_{1A}$ receptor ([21–23]; reviewed in [24,25]).

Lipid–protein interactions can be suitably monitored if the membrane protein in question is purified. An essential criterion for purification of an integral membrane protein is that the protein must be carefully removed from the native membrane and dispersed individually in solution. This is most effectively accomplished using amphiphilic detergents and the process is known as solubilization [26,27]. Solubilization of membrane proteins is a process in which the proteins and lipids, that are held together in native membranes, are suitably dissociated in a buffered detergent solution. The controlled dissociation of the membrane results in the formation of small protein and lipid clusters that remain dissolved in the aqueous solution. We earlier partially purified the hippocampal serotonin$_{1A}$ receptor by solubilizing the receptor in a functionally active form [28] using CHAPS, a mild non-denaturing and zwitterionic detergent. Solubilized membranes represent serotonin$_{1A}$ receptor in relatively purified (enriched) form after loss of other proteins and lipids. In the absence of purified receptors (*none of the G-protein coupled serotonin receptors has been purified from natural sources yet*), functionally active solubilized receptors represent a suitable system to monitor lipid–protein interactions. Solubilization often leads to delipidation, *i.e.*, loss of membrane lipids. This results in considerable loss of activity of the solubilized protein or receptor, since lipid–protein interactions play a crucial role in maintaining the structure and function of integral membrane proteins and receptors [29]. Interestingly, it has been previously shown that solubilization of the serotonin$_{1A}$ receptor by CHAPS leads to reduction in membrane cholesterol and ligand binding function [30,31]. We have earlier shown that ligand binding function of the serotonin$_{1A}$ receptor could be restored upon replenishment of solubilized membranes with cholesterol [31]. In order to examine the stringency of sterol structure necessary for ligand binding function of serotonin$_{1A}$ receptors, we monitored whether desmosterol can support the ligand binding function of the solubilized hippocampal serotonin$_{1A}$ receptor.

2. Materials and methods

2.1. Materials

Desmosterol, CHAPS, cholesterol, MβCD, DMPC, DPH, EDTA, EGTA, $MgCl_2$, $MnCl_2$, iodoacetamide, PEG, PMSF, serotonin, sucrose, polyethylenimine, sodium azide, and Tris were obtained from Sigma Chemical Co. (St. Louis, MO). BCA reagent for protein estimation was from Pierce (Rockford, IL). [^{3}H]8-OH-DPAT (sp. activity 106 Ci/mmol) was purchased from DuPont New England Nuclear (Boston, MA). GF/B glass microfiber filters were from Whatman International (Kent, U.K.). All solvents used were of analytical grade. Precoated silica gel 60 thin layer chromatography plates were from Merck (Darmstadt, Germany). All other chemicals used were of the highest purity available. Water was purified through a Millipore (Bedford, MA) Milli-Q system and used throughout. Fresh bovine brains were obtained from a local slaughterhouse within 10 min of death and the hippocampal region was carefully dissected out. The hippocampi were immediately flash frozen in liquid nitrogen and stored at −70 °C till further use.

2.2. Methods

2.2.1. Preparation of native hippocampal membranes

Native hippocampal membranes were prepared as described previously [32]. Bovine hippocampal tissue (~50 g) was homogenized 10% (w/v) in a polytron homogenizer in buffer A (2.5 mM Tris, 0.32 M sucrose, 5 mM EDTA, 5 mM EGTA, 0.02% sodium azide, 0.24 mM PMSF, 10 mM iodoacetamide, pH 7.4). The homogenate was centrifuged at 900×*g* for 10 min at 4 °C. The resultant supernatant was filtered through four layers of cheesecloth and centrifuged at 50,000×*g* for 20 min at 4 °C. The pellet obtained was suspended in 10 vol. of buffer B (50 mM Tris, 1 mM EDTA, 0.24 mM PMSF, 10 mM iodoacetamide, pH 7.4) using a hand-held Dounce homogenizer and centrifuged at 50,000×*g* for 20 min at 4 °C. This procedure was repeated until the supernatant was clear. The final pellet (native membranes) was suspended in a minimum volume of buffer C (50 mM Tris, pH 7.4), homogenized using a hand-held Dounce homogenizer, flash frozen in liquid nitrogen and stored at −70 °C. Protein concentration was assayed using the BCA reagent [33].

2.2.2. Solubilization of native membranes

Native hippocampal membranes were solubilized as described earlier using CHAPS [28,34]. CHAPS-solubilized membrane was precipitated using PEG in order to remove NaCl from the solubilized extract, since agonist binding of the serotonin$_{1A}$ receptor is inhibited by NaCl [32]. This procedure is also believed to remove detergent. The PEG-precipitated CHAPS-solubilized membrane (referred to as solubilized membranes in this paper) was suspended in buffer C and used immediately either for radioligand binding assays or for sterol replenishment.

2.2.3. Desmosterol and cholesterol replenishment of solubilized membranes

Solubilized membranes were replenished with desmosterol or cholesterol using water soluble desmosterol–MβCD or cholesterol–MβCD complex. The complex was prepared by dissolving required amounts of desmosterol or cholesterol and MβCD in a ratio of 1:10 (mol/mol) in buffer C by constant vortexing at room temperature (~23 °C). Stock solutions (typically 2 mM desmosterol (or cholesterol):20 mM MβCD) of this complex were freshly prepared prior to each experiment. Desmosterol and cholesterol replenishments were carried out at a protein concentration of ~2 mg/ml by incubating solubilized membranes with 1 mM desmosterol (or cholesterol):10 mM MβCD complex for 30 min in buffer C at 25 °C under constant shaking. Membranes were then spun down at 100,000×*g* for 1 h at 4 °C, suspended in the same buffer, and immediately used for radioligand binding assays.

2.2.4. Radioligand binding assays

Receptor binding assays were carried out as described earlier [22] with some modifications. Tubes in duplicate with ~0.5 mg protein in a total volume of 1 ml of buffer D (50 mM Tris, 1 mM EDTA, 10 mM $MgCl_2$, 5 mM $MnCl_2$, pH 7.4) were incubated with the radiolabeled agonist [^{3}H]8-OH-DPAT (final concentration in assay tube being 0.29 nM) for 1 h at 25 °C. Nonspecific binding was determined by performing the assay in the presence of 10 μM serotonin. The binding reaction was terminated by rapid filtration under vacuum in a Millipore multiport filtration apparatus through Whatman GF/B

2.5 cm diameter glass microfiber filters (1.0 μm pore size), which were presoaked in 0.15% polyethylenimine for 1 h [35]. Filters were then washed three times with 3 ml of cold water (4 °C), dried and the retained radioactivity was measured in a Packard Tri-Carb 1500 liquid scintillation counter using 5 ml of scintillation fluid.

2.2.5. Estimation of desmosterol and cholesterol by thin layer chromatography

Total lipids were extracted from native and solubilized membranes, and cholesterol/desmosterol-replenished solubilized membranes according to Bligh and Dyer [36]. Lipid extracts were dried under a stream of nitrogen at 45 °C. The dried extracts were then dissolved in a mixture of chloroform/methanol (1:1, v/v). Sterol contents of membranes under various conditions were estimated by TLC. Precoated silica gel TLC plates were impregnated with 3% (w/v) silver nitrate in methanol, allowed to dry briefly and activated at 120 °C for 15 min. Sterols were resolved using chloroform/diethyl ether (95:5, v/v) as the solvent system as described previously [37]. In order to achieve maximum separation, TLC was run three times in the same solvent and the plate was dried after each run. The separated lipids were visualized by charring with a solution containing cupric sulfate (10%, w/v) and phosphoric acid (8%, v/v) at 150 °C. Desmosterol and cholesterol bands were identified with the help of standards (Fig. 2a). TLC plates were scanned and sterol band intensities were analyzed as described earlier [38].

2.2.6. Estimation of inorganic phosphate

The concentration of lipid phosphate was determined subsequent to total digestion by perchloric acid [39] using Na_2HPO_4 as standard. DMPC was used as an internal standard to assess lipid digestion. Samples without perchloric acid digestion produced negligible readings.

2.2.7. Fluorescence anisotropy measurements

Fluorescence anisotropy measurements were carried out using the fluorescent probe 1,6-diphenyl-1,3,5-hexatriene (DPH) as described previously [34,38]. Steady state fluorescence was measured in a Hitachi F-4010 spectrofluorometer using 1 cm path length quartz cuvettes at room temperature (~23 °C). Excitation and emission wavelengths were set at 358 and 430 nm. Excitation and emission slits with nominal bandpasses of 1.5 and 20 nm were used. The optical density of the samples measured at 358 nm was 0.15 ± 0.01. The anisotropy values remained identical even after dilution of membrane samples, indicating the absence of any scattering artifact [40]. Fluorescence anisotropy measurements were performed using a Hitachi polarization accessory. Anisotropy (r) values were calculated as described previously [37,41]. All experiments were done with multiple sets of samples and average values of fluorescence anisotropy are shown in Fig. 4.

2.2.8. Statistical analysis

Significance levels were estimated by Student's two-tailed unpaired *t*-test using Graphpad Prism software version 4.0 (San Diego, CA).

3. Results and discussion

Effective solubilization and purification of a membrane protein in a functionally active form represent important steps in understanding the structure-function relationship of a given protein [26]. However, solubilization of a membrane protein with retention of activity poses a formidable challenge since many detergents irreversibly denature membrane proteins [42]. It should be mentioned here that the conditions used by us for solubilization of the serotonin$_{1A}$ receptor are fine-tuned and highly optimized. For example, it is known that treatment of membranes with high concentration of CHAPS results in dissociation and depletion of βγ dimer of trimeric G-proteins [43–45].

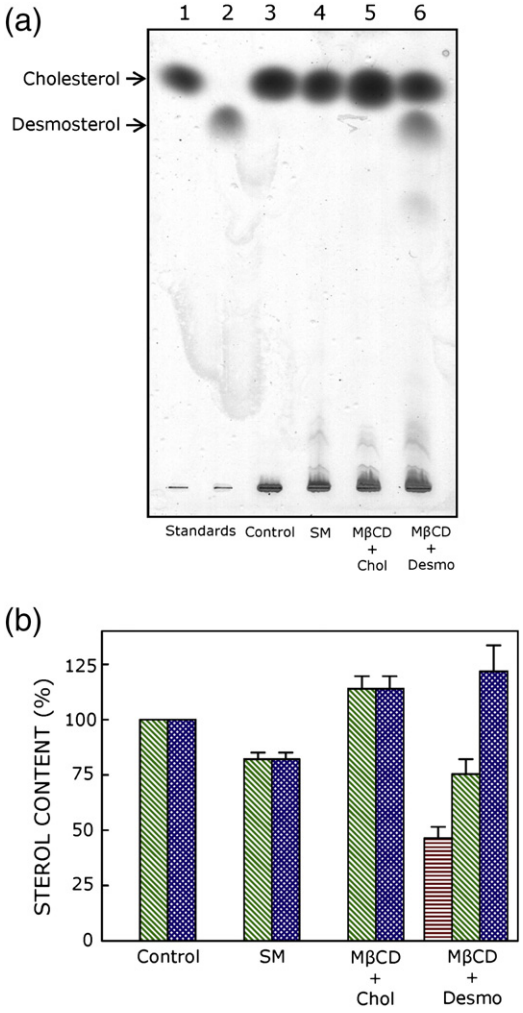

Fig. 2. Estimation of sterol content of native and PEG-precipitated CHAPS-solubilized hippocampal membranes (solubilized membranes, SM), and cholesterol/desmosterol-replenished solubilized membranes. Total lipids were extracted from membranes and sterols were separated using TLC as shown in (a). The chromatogram shows sterols from native membranes (lane 3), solubilized membranes (lane 4), and solubilized membranes replenished with either cholesterol (lane 5) or desmosterol (lane 6). The arrows represent positions of cholesterol and desmosterol on the thin layer chromatogram identified using standards in lanes 1 and 2, respectively. Cholesterol (hatched bar), desmosterol (horizontal lined bar) and total sterol (crisscrossed bar) were quantified by densitometric analysis of the chromatogram and are shown in (b). Values are expressed as percentages of the cholesterol content of native membranes without any treatment and total sterol content of membranes was obtained by the addition of cholesterol and desmosterol contents. Data represent means ± S.E. of three independent experiments. See Materials and methods for more details.

Use of CHAPS at high concentration may therefore be detrimental for solubilizing G-protein coupled receptors in a functionally active form. Keeping this in mind, we devised an efficient strategy using CHAPS at a low (pre-micellar) concentration in presence of NaCl followed by PEG precipitation. The advantage of using low concentration of CHAPS is that the receptor-G protein coupling remains unperturbed. In addition, PEG precipitation helps in efficiently removing detergent and salt from solubilized membranes [46–50]. Taken together, hippocampal membranes solubilized this way represent one of the best membrane system available for exploring lipid–protein interactions.

Solubilization of native membranes results in loss of cholesterol and treatment with MβCD–sterol complex is an effective approach to replenish membranes with sterol [38]. Fig. 2a shows the chromatogram displaying sterol contents in native (control), solubilized and solubilized membranes replenished with either desmosterol or cholesterol.

Cholesterol content in native hippocampal membranes exhibits ~18% reduction upon solubilization (see Fig. 2b). This is accompanied by a corresponding reduction (~36%) in specific [^{3}H]8-OH-DPAT binding to the serotonin$_{1A}$ receptor (see Fig. 3). Subsequent treatment with MβCD–cholesterol complex increased the cholesterol content to ~114% of control membranes (Fig. 2b). This resulted in recovery (~99% of native membranes) of specific [^{3}H]8-OH-DPAT binding (Fig. 3). In order to examine whether replenishment with desmosterol could restore specific [^{3}H]8-OH-DPAT binding, solubilized membranes were treated with MβCD–desmosterol complex. Fig. 2b shows that the extent of replenishment of desmosterol was comparable such that the total sterol (cholesterol + desmosterol) content of desmosterol-replenished membranes was ~122%. The phospholipid content was found to be unaltered under sterol replenishment conditions (see Fig. S1). Interestingly, Fig. 3 shows that specific [^{3}H]8-OH-DPAT binding was completely restored (to ~100% of native membranes), when replenishment was carried out with desmosterol. These results therefore demonstrate that *desmosterol can replace cholesterol* for specific ligand binding function of the hippocampal serotonin$_{1A}$ receptor in solubilized membranes. These results could have important implications in the overall context of sterol binding sites in the serotonin$_{1A}$ receptor (see later).

In order to monitor the overall membrane order under these conditions, fluorescence anisotropy measurements were carried out with the membrane probe DPH. DPH is a rod-like molecule and partitions into the interior of the bilayer. This partitioning has previously been shown to be independent of the phase state of the membrane [51]. Fluorescence anisotropy is correlated to the rotational diffusion of membrane embedded probes such as DPH [41], which is sensitive to the packing of lipid acyl chains. Fig. 4 shows that the fluorescence anisotropy of DPH does not exhibit a significant change in solubilized and desmosterol- or cholesterol-replenished solubilized hippocampal membranes. The relative invariance of the fluorescence anisotropy of DPH could be due to minor changes in sterol/phospholipid ratio under these conditions [52]. This points out that the overall membrane order during solubilization and upon sterol replenishment remains invariant within experimental error.

The function of a growing number of GPCRs depends on membrane cholesterol (a comprehensive list is provided in ref. [25]). Previous work from our laboratory demonstrated the necessity of membrane cholesterol in maintaining the ligand binding function of the hippocampal serotonin$_{1A}$ receptor [22,53,54]. In this paper, we have replaced cholesterol with desmosterol to test the stringency of the requirement of membrane cholesterol in maintaining the function

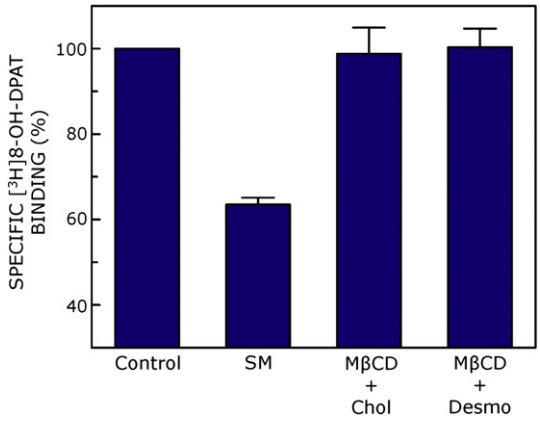

Fig. 3. Effect of replenishment of desmosterol or cholesterol into solubilized membranes on specific binding of [^{3}H]8-OH-DPAT to the hippocampal serotonin$_{1A}$ receptor. Solubilized membranes were replenished with desmosterol or cholesterol, using 1 mM desmosterol (or cholesterol):10 mM MβCD complex. Values are expressed as percentages of specific binding obtained in native membranes. Data shown are means ± S.E. from at least five independent experiments. See Materials and methods for other details.

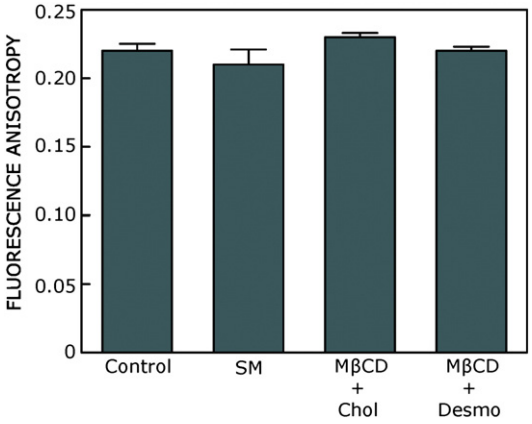

Fig. 4. Effect of replenishment of cholesterol and desmosterol into solubilized membranes on steady state fluorescence anisotropy of the membrane probe DPH. Solubilized membranes were replenished with desmosterol or cholesterol, using 1 mM desmosterol (or cholesterol):10 mM MβCD complex. Fluorescence anisotropy measurements were performed with membranes containing 50 nmol phospholipid at a probe to phospholipid ratio of 1:100 (mol/mol) at room temperature (~23 °C). Values represent means ± S.E. of duplicate points from three independent experiments. See Materials and methods for more details.

of the *solubilized* hippocampal serotonin$_{1A}$ receptor. As mentioned earlier, desmosterol differs with cholesterol only in a double bond at the 24th position in the flexible acyl side chain. Our results demonstrate that desmosterol is capable of supporting the ligand binding function of the solubilized serotonin$_{1A}$ receptor.

Previous biophysical measurements by us [55] and others [56,57] have shown that the effects of cholesterol and desmosterol on membrane organization and dynamics are similar. Desmosterol has previously been shown to substitute for cholesterol in mutant mouse L-cell fibroblasts [58]. In addition, it has been shown that in absence of cholesterol, desmosterol alone can support cell proliferation in a murine macrophage-like cells [59]. More importantly, results from studies using *Dhcr24* gene knockout (*Dhcr24*$^{-/-}$) mice [12] or cells derived from them [60,61] show that desmosterol can act as a suitable replacement for cholesterol. For example, the *Dhcr24* gene knockout mice are viable and exhibit a mild phenotype although they are smaller in size and are sterile [12]. However, certain degree of caution should be exercised while interpreting these results. The *Dhcr24*$^{-/-}$ mice are not totally cholesterol-free (strictly speaking, these are low cholesterol, high desmosterol *Dhcr24*$^{-/-}$ knockout mice) due to availability of maternal cholesterol in mice during embryogenesis [12] since maternal cholesterol can cross the placenta in rodents [62]. Our results showing that desmosterol is capable of supporting the ligand binding function of the solubilized serotonin$_{1A}$ receptor is in overall agreement with the above reports.

Interestingly, we have previously shown that desmosterol is not capable of supporting the ligand binding function of the hippocampal serotonin$_{1A}$ receptor *when sterol (cholesterol) depletion and replenishment are carried out using MβCD and MβCD–sterol complex, respectively* [37]. Our present results appear to be contradictory to these results. We believe that an analysis of the reason for this apparent discrepancy could provide novel information about the nature of cholesterol binding sites around the serotonin$_{1A}$ receptor. The key difference in these instances is the way we have carried out sterol manipulations in the membrane. While in our earlier work, we used MβCD for such sterol manipulations [37], we employed the strategy of solubilization and effectively utilized the lipid loss associated with solubilization [30,31] for achieving sterol depletion in the present work.

We recently proposed that cholesterol binding sites in GPCRs could represent nonannular binding sites [63]. Integral membrane proteins are surrounded by a shell or annulus of lipid molecules,

which mimics the immediate layer of solvent surrounding soluble proteins [64]. These are termed 'annular' lipids surrounding the membrane protein. The annular lipids are exchangeable with bulk lipids [65], although this exchange rate is slower compared to exchange rate between bulk lipids. In addition to the annular lipids, there is evidence for other lipid molecules in the immediate vicinity of integral membrane proteins. These are termed as 'nonannular' lipids (see Fig. 5a). Cholesterol has previously been proposed to be localized in nonannular sites in Ca^{2+}/Mg^{2+}-ATPase [66], the nicotinic acetylcholine receptor [67], and anionic phospholipids have been reported to occupy nonannular sites in the potassium channel KcsA from *Streptomyces lividans* [68] and gap junction hemichannel connexin 26 [69]. Nonannular sites are characterized by lack of accessibility to the annular lipids, *i.e.*, these sites cannot be displaced by competition with annular lipids. Binding to the nonannular sites is considered to be more specific compared to annular binding sites [64]. As a result, nonannular lipid binding sites remain vacant even in the presence of annular lipids around the protein [68]. It has been suggested that the possible locations for the nonannular sites could be either inter or intramolecular (interhelical) protein interfaces, characterized as deep clefts (or cavities) on the protein surface [66,68].

Nonannular lipids are often believed to be preserved in the high resolution crystal structure of membrane proteins, *i.e.*, they survive the crystallization conditions [64]. Lipid molecules resolved in high

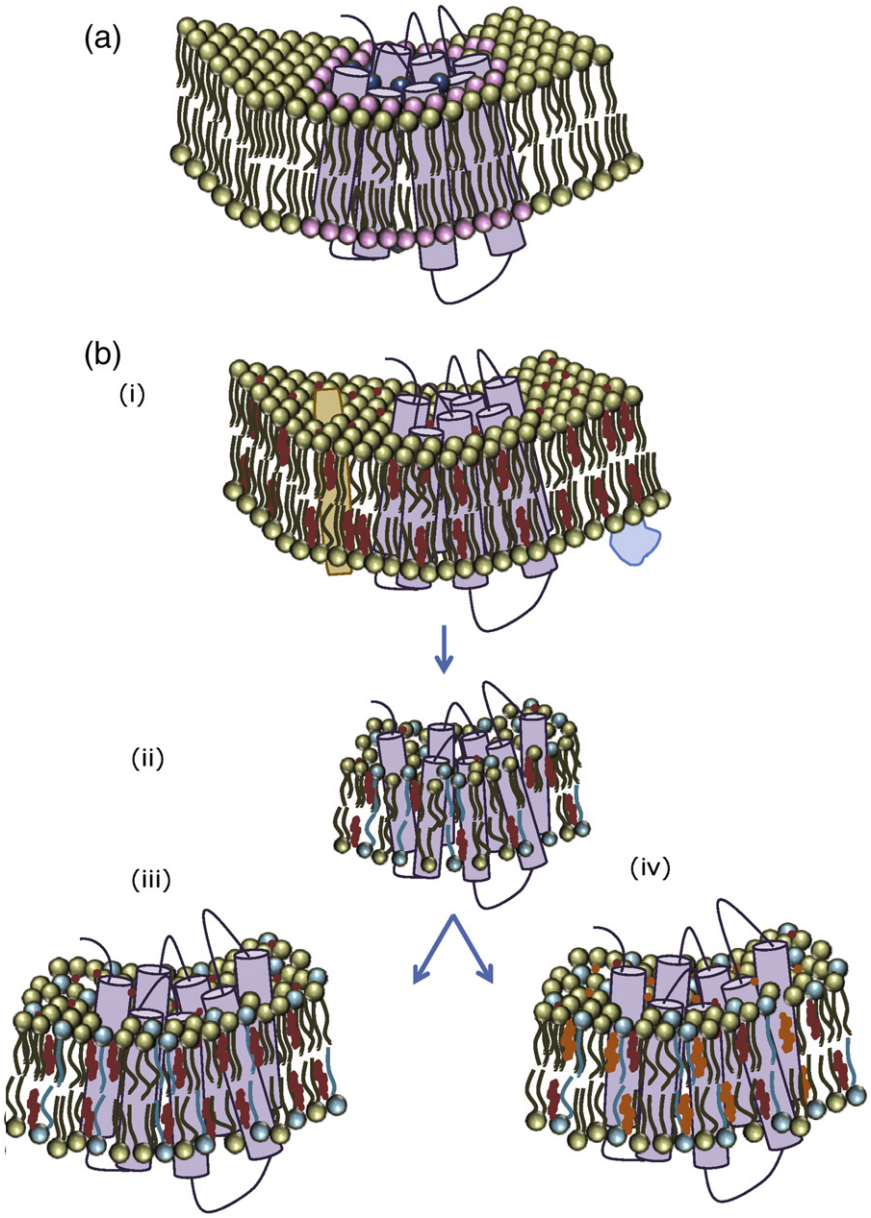

Fig. 5. (a) A schematic representation of a membrane embedded seven transmembrane domain receptor showing various classes of lipids in the vicinity of the receptor. Annular lipids (shown in pink) represent the shell (or annulus) of lipid molecules around the receptor. Nonannular lipids (shown in blue) are characterized by lack of accessibility to annular lipids. Bulk lipids are shown in green. See text for more details. (b) A schematic representation depicting (i) native membranes, (ii) solubilized membranes, (iii) cholesterol-replenished solubilized membranes, and (iv) desmosterol-replenished solubilized membranes. Native membranes in (i) show phospholipids (green), cholesterol (maroon), the serotonin$_{1A}$ receptor (purple), other integral (light brown) and peripheral (light blue) membrane proteins. (ii) Solubilization of native membranes with the zwitterionic detergent CHAPS (single tailed molecule in cyan), results in loss of phospholipids, and integral and peripheral membrane proteins, leading to partial purification of serotonin$_{1A}$ receptors. Solubilization also causes loss of cholesterol bound to the serotonin$_{1A}$ receptor and appears to affect the 'nonannular' binding sites for cholesterol. Replenishment of solubilized membranes with (iii) cholesterol and (iv) desmosterol (shown in orange) appears to restore nonannular lipid sites resulting in recovery of specific ligand binding to the serotonin$_{1A}$ receptor. Note that the membrane components are loosely packed in the solubilized membrane. See text for more details.

resolution crystal structures of membrane proteins are therefore likely to be nonannular lipids [29]. We have recently suggested, based on the reported crystal structure of the β_2-adrenergic receptor [70,71], that cholesterol molecules located at the interhelical/interprotein regions of the receptor could represent nonannular lipids [63]. Based on the high degree of sequence similarity (~48%) between transmembrane regions of the serotonin$_{1A}$ receptor and β_2-adrenergic receptor [72], we propose that the cholesterol-dependent activity of the serotonin$_{1A}$ receptor is partly due to the presence of tightly bound cholesterol molecules at the nonannular site(s). In fact, we have recently reported from receptor modeling studies that the serotonin$_{1A}$ receptor is more compact in the presence of tightly bound cholesterol [72]. In addition, we have very recently identified cholesterol recognition/interaction amino acid consensus (CRAC) motifs in the serotonin$_{1A}$ receptor [73]. The CRAC motif represents a characteristic structural feature of proteins that are believed to result in preferential association with cholesterol [74,75]. As mentioned above, it has been previously postulated that the possible locations for the nonannular sites could be either inter or intramolecular (interhelical) protein interfaces, characterized as deep clefts (or cavities) on the protein surface [66,67]. Interestingly, it has been suggested that cholesterol binding by CRAC motif is induced by a similar cleft located at the membrane interfacial region [76]. Taken together, nonannular cholesterol appears to be crucial for maintaining the function of the serotonin$_{1A}$ receptor.

We believe that the difference in results obtained in the above two cases lies in the ability of desmosterol to occupy nonannular sites when added as MβCD–desmosterol complex following solubilization since solubilization allows a more robust reorganization of membranes (see Fig. 5b). This is because membrane lipids and proteins are loosely packed in solubilized membranes compared to MβCD-treated native membranes. Solubilized membranes are loose, metastable and are composed of heterogeneous complexes of detergent, lipid and protein, forming mixed micelles [26]. We previously attributed such loose packing of solubilized membranes to enhanced susceptibility of the serotonin$_{1A}$ receptor to ethanol [77]. MβCD, on the other hand, is known to preferentially deplete membrane cholesterol from regions of the membrane where cholesterol is present in relatively fluid (liquid disordered) phase and not from the ordered cholesterol-rich domains [78–80]. Membrane reorganization as a result of such cholesterol depletion by MβCD therefore may not be sufficient (robust) to replace the nonannular sites with desmosterol, a necessary step for recovery of activity. Solubilization and subsequent sterol replenishment may just allow this important step, thereby resulting in recovery of activity (Fig. 3).

In summary, we show here that desmosterol is capable of supporting the ligand binding function of the solubilized serotonin$_{1A}$ receptor, provided sterol manipulation is carried out on solubilized membranes. Our results are consistent with previous reports, spanning biophysical studies using model membranes [55–57] to animal models [12,60,61], showing that desmosterol can replace cholesterol in a large number of cases. We further conclude that caution should be exercised while interpreting results from sterol replacement experiments, in view of the complexities involved.

Supplementary materials related to this article can be found online at doi:10.1016/j.bbamem.2011.06.022.

Acknowledgments

This work was supported by the Council of Scientific and Industrial Research, Government of India. P.S. and Y.D.P. thank the Council of Scientific and Industrial Research for the award of Senior Research Fellowships. A.C. is an Adjunct Professor at the Special Centre for Molecular Medicine of Jawaharlal Nehru University (New Delhi, India) and Indian Institute of Science Education and Research (Mohali, India), and Honorary Professor at the Jawaharlal Nehru Centre for Advanced Scientific Research (Bangalore, India). A.C. gratefully acknowledges J.C. Bose Fellowship (Department of Science and Technology, Government of India). We gratefully acknowledge Roopali Saxena for helpful discussion and help in creating Fig. 5. We thank members of our laboratory for critically reading the manuscript.

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